Express Mail Mailing Label No. EJ606935626US

FORM PTO-1390 (REV 12-29-99)

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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 0263-4047

US APPLICATION NO (If known, see 37 CFR 1 5): 09/622487

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/JP99/01080	05 March 1999 (05.03.99)	06 March 1998 (06.03.98)

TITLE OF INVENTION: PROTEIN-FREE FORMULATIONS

424 Rec'd PCT/PTO 17 AUG 2000

APPLICANT(S) FOR DO/EO/US: Shuji SUMIDA and Yasushi SATO

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [X] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [X] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [X] A translation of the International Application into English (35 U.S.C. 371(c)(2)). and Verification of a Translation
7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). and Power of Attorney, duly executed
10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. [X] An Information Disclosure Statement under 37 CFR 1.97 and 1.98. with a copy of the International Search Report
12. [X] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [ ] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [x] Other items or information: Copy of Form PCT/RO/101, PCT Request as filed 05.03.1999;
    Copies of Forms PCT/IB/304, PCT/IB/308 and PCT/IB/332;
    Copy of the first page of PCT Publication No. WO99/44630;
    Copy of the International Preliminary Examination Report (in Japanese);
    Copy of the International Search Report;
    Verified Certification of Express Mailing Date (International Application) 37 C.F.R. 1.10;
    3 sheets of formal drawings (labeled); and
    Return Receipt Postcard.

U.S. APPLICATION NO (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
09/622487	PCT/JP99/01080	0263-4047

17. [X] The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :	CALCULATIONS PTO USE ONLY
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$970.00**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$840.00**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$690.00**	
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$670.00**	
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **$96.00**	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 840.00
Surcharge of **$130.00** for furnishing the oath or declaration later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ --

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	12 - 20 =	--	X **$18.00**	$ --
Independent claims	1 - 3 =	--	X **$78.00**	$ --
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$260.0**	$ --
TOTAL OF ABOVE CALCULATIONS =				$ 840.00
Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statement must also by filed (Note 37 CFR 1.9, 1.27, 1.28).				$ --
SUBTOTAL =				$ 840.00
Processing fee of **$130.00** for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$ --
TOTAL NATIONAL FEE =				$ 840.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$ 40.00
TOTAL FEES ENCLOSED =				$ 880.00
			Amount to be refunded:	$ --
			charged:	$ --

a. [X] A check in the amount of $ 840.00 to cover the above fees is enclosed.
A check in the amount of $40.00 to cover the fee for recoring the Assignment is enclosed.

b. [ ] Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 13-4500. A duplicate copy of this sheet is enclosed.

Order No. 0263-4047

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO
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SIGNATURE

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Reg. No. 25,237
REGISTRATION NUMBER

VERIFICATION OF A TRANSLATION

I, the below named translator, hereby declare that:

My name and post office address are as stated below;

That I am knowledgeable in the English language and in the language in which the below identified application was filed, and that I believe the English translation of International Application No. PCT/JP99/01080 is a true and complete translation of the above identified International Application as filed.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated this 4th day of August, 2000

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SPECIFICATION

PROTEIN-FREE FORMULATIONS

FIELD OF THE INVENTION

The present invention relates to granulocyte colony-stimulating factor-containing formulations, and particularly granulocyte colony-stimulating factor-containing formulations stabilized by preventing loss and inactivation of active ingredients due to adsorption to container walls, aggregation, polymerization, oxidation or the like.

PRIOR ART

Granulocyte colony-stimulating factor (hereinafter also referred to as "G-CSF") is a glycoprotein having a molecular weight of about 20,000 and acting on precursor cells of neurophils to promote their proliferation and differentiation to maturation.

Since we purified high-purity human G-CSF by culturing a cell line collected from tumor cells of a patient with cancer of the floor of the mouth, the human G-CSF gene was successfully cloned and, at present, recombinant human G-CSF can be produced in mass in animal cells by genetic engineering. We also succeeded in converting this purified G-CSF into a formulated product, which is supplied to the market as an antiinfective agent (Japanese Patent No. 2116515).

G-CSF is used in a very small amount, i.e. a formulation containing 0.1-1000 μg (preferably 5-500 μg) of G-CSF is normally administered once to seven times per week

per adult. However, this G-CSF is adsorptive to walls of ampoules, syringes or the like. G-CSF is also unstable and susceptible to extrinsic factors such as temperature, humidity, oxygen, UV rays or the like to undergo physical or chemical changes including aggregation, polymerization or oxidation, resulting in great loss of activity.

Thus, various formulation designs have been made to supply stable G-CSF formulations to the market. For example, formulations containing a buffer selected from acetic acid, lactic acid, citric acid, maleic acid, phosphoric acid and salts thereof or arginine and salts thereof were proposed (JPA No. 505610/96). G-CSF formulations containing 1-10,000 parts by weight of a surfactant as a stabilizer per part by weight of G-CSF were also proposed (JPA No. 146826/88). The latter publication describes that the level of the surfactant, particularly its lower limit is critical to prevent loss of G-CSF and to achieve stabilization in G-CSF-containing liquid formulations.

An object of the present invention is to provide a G-CSF formulation, which enables a reduction in the complexity of the production process and which is more stable for extended storage.

SUMMARY OF THE INVENTION

As a result of careful studies to achieve the above object, we accomplished the present invention on the basis of the finding that a stable G-CSF liquid formulation can be obtained even when it contains a very small amount of a

surfactant as a stabilizer.

Accordingly, the present invention provides a stable granulocyte colony-stimulating factor-containing formulation comprising a granulocyte colony-stimulating factor and 0.0001-1 parts by weight of at least one pharmaceutically acceptable surfactant per part by weight of the granulocyte colony-stimulating factor and having a pH of 7 or less.

As used herein, stabilization means that the percentage of remaining G-CSF is kept at 95% or more after storage at 25°C for 6 months or 75% or more after storage at 40°C for 2 weeks.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph showing the relationship between pH and the percentage of remaining G-CSF after an acceleration test at 40°C for 2 weeks.

FIG. 2 is a graph showing the relationship between pH and the production ratio of desialylated G-CSF after an acceleration test at 40°C for 2 weeks.

FIG. 3 is a graph showing the relationship between the part by weight of Polysorbate 20 per part by weight of G-CSF and the adsorption inhibition rate after the lapse of 24 hours after packing.

THE MOST PREFERRED EMBODIMENTS OF THE INVENTION

Any high-purity human G-CSF may be used for liquid formulations of the present invention. Specifically, it may be derived from natural sources or obtained by genetic recombination so far as it has substantially the same

biological activity as that of mammalian, particularly human G-CSF. Genetically recombinant G-CSF may have the same amino acid sequence as that of natural G-CSF or may contain deletion, substitution or addition of one or a plurality of amino acids in said amino acid sequence so far as it has said biological activity. G-CSF in the present invention may be prepared by any process, e.g., they may be extracted and purified by various techniques from cultures of a human tumor cell line or may be produced by genetic engineering in cells of E. coli, yeast, Chinese hamster ovary (CHO), C127 or the like and then extracted and purified by various techniques. Most preferably, G-CSF is produced in CHO cells by genetic recombination.

Typical examples of surfactants suitable for obtaining stable G-CSF-containing formulations of the present invention include nonionic surfactants, e.g., sorbitan fatty acid esters such as sorbitan monocaprylate, sorbitan monolaurate, sorbitan monopalmitate; glycerin fatty acid esters such as glycerin monocaprylate, glycerin monomyristate, glycerin monostearate; polyglycerin fatty acid esters such as decaglyceryl monostearate, decaglyceryl distearate, decaglyceryl monolinoleate; polyoxyethylene sorbitan fatty acid esters such as polyoxyethylene sorbitan monolaurate, polyoxyethylene sorbitan monooleate, polyoxyethylene sorbitan monostearate, polyoxyethylene sorbitan monopalmitate, polyoxyethylene sorbitan trioleate, polyoxyethylene sorbitan tristearate; polyoxyethylene sorbitol fatty acid esters such as polyoxyethylene sorbitol

tetrastearate, polyoxyethylene sorbitol tetraoleate; polyoxyethylene glycerin fatty acid esters such as polyoxyethylene glyceryl monostearate; polyethylene glycol fatty acid esters such as polyethylene glycol distearate; polyoxyethylene alkyl ethers such as polyoxyethylene lauryl ether; polyoxyethylene polyoxypropylene alkyl ethers such as polyoxyethylene polyoxypropylene glycol ether, polyoxyethylene polyoxypropylene propyl ether, polyoxyethylene polyoxypropylene cetyl ether; polyoxyethylene alkyl phenyl ethers such as polyoxyethylene nonyl phenyl ether; polyoxyethylene hardened castor oils such as polyoxyethylene castor oil, polyoxyethylene hardened castor oil (polyoxyethylene hydrogenated castor oil); polyoxyethylene beeswax derivatives such as polyoxyethylene sorbitol beeswax; polyoxyethylene lanolin derivatives such as polyoxyethylene lanolin; polyoxyethylene fatty acid amides such as polyoxyethylene stearic acid amide having a HLB of 6-18; cationic surfactants, e.g., alkyl sulfates having a C10-18 alkyl group such as sodium cetylsulfate, sodium laurylsulfate, sodium oleylsulfate; polyoxyethylene alkyl ether sulfates having an average number of EO moles of 2-4 and a C10-18 alkyl group such as sodium polyoxyethylene laurylsulfate; alkyl sulfosuccinic acid ester salts having a C8-18 alkyl group such as sodium laurylsulfosuccinate; natural surfactants, e.g., lecithin; grycerophospholipids; sphingophospholipids such as sphingomyelin; sucrose fatty acid esters of fatty acids containing 12 to 18 carbon atoms.

One or two or more of these surfactants may be added to liquid formulations of the present invention.

Preferred surfactants are polyoxyethylene sorbitan fatty acid esters, more preferablly Polysorbates 20, 21, 40, 60, 65, 80, 81, 85, most preferably Polysorbates 20 and 80.

The amount of surfactants to be added to G-CSF-containing formulations of the present invention is typically 0.0001-1 parts by weight per part by weight of G-CSF, preferably 0.01-1 parts by weight per part by weight of G-CSF, more preferably 0.2-1 parts by weight per part by weight of G-CSF, even more preferably 0.2-0.8 parts by weight per part by weight of G-CSF, most preferably 0.4-0.8 parts by weight per part by weight of G-CSF. Particularly when 125 μg or 250 μg of G-CSF is contained per mL of formulations, 100 μg of surfactants are preferably added. Therefore, 0.4 parts by weight or 0.8 parts by weight of surfactants are especially preferred per part by weight of G-CSF. When any protein such as albumin is not added as a stabilizer, the percentage of remaining G-CSF tended to decrease after extended storage in the presence of surfactants exceeding 1 part by weight per part by weight of G-CSF. Even 1 part by weight or less of surfactants per part by weight of G-CSF can sufficiently inhibit adsorption of G-CSF to containers.

Preferred G-CSF-containing formulations of the present invention are substantially free from protein as a stabilizer. Some products on the market contain a protein such as human serum albumin or purified gelatin as a

stabilizer for inhibiting chemical or physical changes of G-CSF. However, the addition of a protein as a stabilizer involves a very complicated process for removing contamination with viruses or other problems.

G-CSF-containing formulations of the present invention have a pH of 7 or less, preferably 5-7, more preferably 6-6.8, most preferably 6.2-6.8. As will be described later, the percentage of remaining G-CSF after an acceleration test at 40°C for 2 weeks is stable at a pH of 7 or less. From this viewpoint, the pH is preferably about 7.0 or less. The production ratio of desialylated G-CSF determined after an acceleration test at 40°C for 2 weeks showed a rapid increase of the content of desialylated products at pH 4 or less. From this viewpoint, the pH is preferably about 5 or more. Further taking into account the preference for neutrality which is less irritable to human bodies for administration as injection formulations, the pH is most preferably 6.2-6.8.

G-CSF-containing formulations of the present invention may contain diluents, solubilizing agents, isotonizing agents, excipients, pH-modifiers, soothing agents, sulfur-containing reducing agents, antioxidants or the like. For example, isotonizing agents include polyethylene glycol; and sugars such as dextran, mannitol, sorbitol, inositol, glucose, fructose, lactose, xylose, mannose, maltose, sucrose, raffinose. Surfur-containing reducing agents include N-acetylcysteine, N-acetylhomocysteine, thioctic acid, thiodiglycol, thioethanolamine, thioglycerol,

thiosorbitol, thioglycolic acid and salts thereof, sodium thiosulfate, glutathione, and sulfhydryl-containing compounds such as thioalkanoic acid having 1 to 7 carbon atoms. Antioxidants include erythorbic acid, dibutylhydroxytoluene, butylhydroxyanisole, α-tocopherol, tocopherol acetate, L-ascorbic acid and salts thereof, L-ascorbyl palmitate, L-ascorbyl stearate, sodium bisulfite, sodium sulfite, triamyl gallate, propyl gallate or chelating agents such as ethylenediamine tetraacetic acid disodium salt (EDTA), sodium pyrophosphate, sodium metaphosphate. As excipients may be added amino acids such as glycine, cysteine, threonine, cystine, tryptophan, methionine, lysine, hydroxylysine, hystidine, arginine. Other components commonly added to liquid formulations may also be contained, e.g. inorganic salts such as sodium chloride, potassium chloride, calcium chloride, sodium phosphate, potassium phosphate, sodium bicarbonate; and organic salts such as sodium citrate, potassium citrate, sodium acetate.

The amount of G-CSF contained in liquid formulations of the present invention depends on the nature of the disease to be treated, the severity of the disease, the age of the patient or other factors, but generally ranges from 1 to 1000 μg/mL, preferably 10 to 800 μg/mL, more preferably 50 to 500 μg/mL.

Liquid formulations of the present invention can be prepared by dissolving these components in an aqueous buffer known in the art of liquid formulations such as

phosphate and/or citrate buffers. Preferred phosphate buffers are sodium monohydrogen phosphate - sodium dihydrogen phosphate series, and preferred citrate buffers are sodium citrate buffers.

Stabilized G-CSF-containing formulations of the present invention are normally administered via parenteral routes such as injection (subcutaneous, intravenous or intramuscular injection) or percutaneous, mucosal, nasal or pulmonary administration, but may also be orally administered.

G-CSF-containing formulations of the present invention are normally packed in a sealed and sterilized plastic or glass container. The container may be provided as a defined dosage form, such as an ampoule, vial or disposable syringe, or may be provided as a large dosage form such as a bag or bottle for injection. Preferably, G-CSF-containing formulations are provided as a dosage form packed in a vial, ampoule or prefilled syringe.

G-CSF-containing formulations of the present invention show a very good percentage of remaining G-CSF even after an acceleration test at 40°C for 2 weeks or storage at 25°C for 6 months as shown in the examples below. The sugar chain of G-CSF has one or two terminal sialic acids, which may be cleaved during extended storage. G-CSF-containing formulations of the present invention were found to keep a low production ratio of desialylated products even after an acceleration test at 40°C for 2 weeks. Moreover, G-CSF-containing formulations of the present invention can

sufficiently inhibit adsorption to containers and show a very good percentage of remaining G-CSF after an acceleration test at 40°C for 2 weeks and after storage at 25°C for 6 months irrespective of the shape of the container such as a vial or syringe.

The following examples further illustrate the present invention, without limiting the same thereto.

EXAMPLES

Experimental Procedure

A mixture of 250mg of G-CSF, 0.1 g of Polysorbate 20 and 30 g of D-mannitol was weighed and adjusted to various pHs shown in the following Table 1 with a sodium phosphate buffer, and then brought to a total amount of 1 L.

Table 1

pH	G-CSF	Polysorbate 20	Mannitol	Sodium phosphate buffer	Total amount
4.0	250 mg	0.1 g	30 g	Equivalent to 25 mM	1 L
5.0	250 mg	0.1 g	30 g	ditto	1 L
5.5	250 mg	0.1 g	30 g	ditto	1 L
6.0	250 mg	0.1 g	30 g	ditto	1 L
6.5	250 mg	0.1 g	30 g	ditto	1 L
7.0	250 mg	0.1 g	30 g	ditto	1 L
7.5	250 mg	0.1 g	30 g	ditto	1 L
8.0	250 mg	0.1 g	30 g	ditto	1 L

Each formulated solution was sterilely prepared and filtrated, after which 1 mL each was sterilely packed into

a vial and sealed to prepare a G-CSF liquid formulation.

Thus sterilely prepared formulation containing 250 μg/mL of G-CSF was allowed to stand in an incubator at 40°C for 2 weeks.

The content of G-CSF in each vial was determined according to the following method 1. The content of desialylated G-CSF in each vial was determined according to the following method 2.

Method 1

Pure water, acetonitrile and trifluoroacetic acid were used as mobile phase on a C4 reverse phase column (4.6 mm x 250 mm, 300 angstroms). The content of G-CSF was determined by reverse phase high-performance liquid chromatography. The amount equivalent to 5 μg of G-CSF was injected and G-CSF was eluted with an acetonitrile gradient and spectroscopically detected at a wavelength of 215 nm.

The G-CSF content determined by this method was used to calculate the remaining percentage (%) after acceleration at 40°C for 2 weeks according to the following equation.

Remaining percentage (%) = [(G-CSF content after acceleration at 40°C for 2 weeks) / (G-CSF content without acceleration)] x 100

Method 2

Desialylated G-CSF (with all the sialic acids of the sugar chain cleaved) and G-CSF (intact) were detected by cation exchange high-performance liquid chromatography.

Namely, both were eluted with an NaCl gradient (0-500 mM) on a cation exchange column (TSK gel DEAE) using 20 mM Tris-HCL buffer (pH 7.4) as mobile phase and spectroscopically detected at a wavelength of 215 nm.

The values of desialylated G-CSF and intact G-CSF determined by this method were used to calculate the production ratio (%) of desialylated G-CSF after acceleration at 40°C for 2 weeks according to the following equation.

Production ratio of desialylated G-CSF (%) = {(desialylated G-CSF) / [(desialylated G-CSF) + (intact G-CSF)]} x 100

Example 1: Effect of varying pHs on the percentage of remaining G-CSF

The percentage of remaining G-CSF was calculated according to the equation of Method 1 after the liquid formulations prepared at varying pHs shown in Table 1 were subjected to an acceleration test at 40°C for 2 weeks. The results are shown in Fig. 1.

At a pH of 7 or less, the percentage of remaining G-CSF was 75% or more.

Example 2: Effect of varying pHs on the production of desialylated G-CSF

The production ratio of desialylated G-CSF was calculated according to the equation of Method 2 after the liquid formulations prepared at varying pHs shown in Table 1 were subjected to an acceleration test at 40°C for 2 weeks. The results are shown in Fig. 2.

At a pH within the range of about 5 to 7, the production ratio of desialylated G-CSF was very low.

Example 3: Effect of the concentration of surfactants on the adsorption of G-CSF to containers

To a mixture of 250 mg of G-CSF and 5.844 g of sodium chloride was added Polysorbate 20 to the concentrations shown in the following Table 2 and the mixture was adjusted at pH 6.5 with a sodium phosphate buffer and brought to a total amount of 1 L.

Table 2

Polysorbate 20	G-CSF	Sodium chloride	Sodium phosphate buffer	pH	Total amount
0 g	250 mg	5.844 g	Equivalent to 15 mM	6.5	1 L
0.02 g	250 mg	5.844 g	ditto	6.5	1 L
0.05 g	250 mg	5.844 g	ditto	6.5	1 L
0.1 g	250 mg	5.844 g	ditto	6.5	1 L
0.2 g	250 mg	5.844 g	ditto	6.5	1 L
0.5 g	250 mg	5.844 g	ditto	6.5	1 L

Each G-CSF formulated solution shown in Table 2 was sterilely prepared and filtrated, after which 1 mL each was sterilely packed into a vial (untreated white glass vial (5 mL) made by Murase Glass) and the G-CSF content was determined by reverse phase high-performance liquid chromatography described in Method 1 immediately after packing and after the lapse of 24 hours after packing.

The G-CSF content determined by this method was used to

calculate the adsorption inhibition rate (%) after the lapse of 24 hours after packing according to the following equation.

Adsorption inhibition rate (%) = [(G-CSF content after the lapse of 24 hr after packing) / (G-CSF content immediately after packing)] x 100

The results are shown in Table 3 and Fig. 3.

Table 3

Parts by weight*)	Adsorption inhibition rate
0	94.5%
0.08	95.0%
0.2	97.5%
0.4	97.0%
0.8	100%
2	99.5%

*) Parts by weight: Parts by weight of Polysorbate 20 per part by weight of G-CSF

Adsorption inhibition rates were sufficient even when the Polysorbate concentration was 1 part by weight or less per part by weight of G-CSF.

Example 4: Stability of formulations packed in a vial or syringe

A formulated solution containing 250 mg of G-CSF, 0.1 g of Polysorbate 20 and 7 g of sodium chloride in a total amount of 1 L and adjusted to pH 6.5 with a sodium phosphate buffer was sterilely prepared and filtrated,

after which 1 mL each was sterilely packed into a vial (see above) or syringe (Hypac SFC, 1 mL long, made by Nippon Becton Dickinson & Co,. Ltd.) and sealed to prepare a G-CSF liquid formulation shown in Table 4.

Table 4

G-CSF	Polysorbate 20	Sodium chloride	Sodium phosphate buffer	pH	Total amount
250 mg	0.1 g	7.0 g	15 mM	6.5	1 L

The thus sterilely prepared formulation containing 250 μg/mL of G-CSF was allowed to stand in an incubator at 40°C for 2 weeks or in an incubator at 25°C for 6 months.

The content of G-CSF in each vial or syringe was determined according to Method 1, and the percentage of remaining G-CSF after acceleration at 40°C for 2 weeks and the percentage of remaining G-CSF after storage at 25°C for 6 months were calculated according to the equation of Method 1.

The results are shown in Table 5.

Table 5

Container type	Remaining percentage	
	Acceleration at 40°C, 2 weeks	Storage at 25°C, 6 months
Vial	89.6%	98.4%
Syringe	90.7%	97.9%

G-CSF formulations of the present invention showed excellent stability, as demonstrated by the remaining

percentage of 75% or more after acceleration at 40°C for 2 weeks and the remaining percentage of 95% or more after storage at 25°C for 6 months in both vial and syringe.

INDUSTRIAL APPLICABILITY

G-CSF-containing formulations of the present invention, which contain a very small amount of surfactants such as 1 part by weight or less per part by weight of G-CSF, can effectively solve the problems related to loss of active ingredients or decrease of activity due to aggregation, polymerization, oxidation or adsorption to container walls caused by extrinsic factors such as the temperature of G-CSF present in a small amount in the formulations, humidity, oxygen, UV rays, etc. Therefore, the present invention provides a liquid formulation which reduces complexity and costs in the production process and which is stable during even extended storage.

CLAIMS

1. A stable granulocyte colony-stimulating factor-containing formulation comprising a granulocyte colony-stimulating factor and at least one pharmaceutically acceptable surfactant in an amount of 1 part by weight or less per part by weight of the granulocyte colony-stimulating factor and having a pH of 7 or less.

2. The granulocyte colony-stimulating factor-containing formulation of Claim 1 wherein the surfactant is contained in an amount ranging from 0.2 to 1 parts by weight per part by weight of the granulocyte colony-stimulating factor.

3. The granulocyte colony-stimulating factor-containing formulation of Claim 2 wherein the surfactant is contained in an amount ranging from 0.2 to 0.8 parts by weight per part by weight of the granulocyte colony-stimulating factor.

4. The granulocyte colony-stimulating factor-containing formulation of Claim 2 wherein the surfactant is contained in an amount ranging from 0.4 to 0.8 parts by weight per part by weight of the granulocyte colony-stimulating factor.

5. The granulocyte colony-stimulating factor-containing formulation of Claim 2 wherein the surfactant is contained in an amount of 0.4 or 0.8 parts by weight per part by weight of the granulocyte colony-stimulating factor.

6. The granulocyte colony-stimulating factor-containing formulation of Claim 1, which is substantially free from protein as a stabilizer.

7. The granulocyte colony-stimulating factor-containing formulation of Claim 1 wherein the surfactant is at least

one member selected from the group consisting of nonionic surfactants such as sorbitan fatty acid esters, glycerin fatty acid esters, polyglycerin fatty acid esters, polyoxyethylene sorbitan fatty acid esters, polyoxyethylene sorbitol fatty acid esters, polyoxyethylene glycerin fatty acid esters, polyethylene glycol fatty acid esters, polyoxyethylene alkyl ethers, polyoxyethylene polyoxypropylene alkyl ethers, polyoxyethylene alkyl phenyl ethers, polyoxyethylene hardened castor oils, polyoxyethylene beeswax derivatives, polyoxyethylene lanolin derivatives, polyoxyethylene fatty acid amides; cationic surfactants such as alkyl sulfates, polyoxyethylene alkyl ether sulfates, alkyl sulfosuccinic acid ester salts; and natural surfactants such as lecithin, grycerophospholipids, sphingophospholipids, sucrose fatty acid esters.

8. The granulocyte colony-stimulating factor-containing formulation of Claim 1 wherein the surfactant is a polyoxyethylene sorbitan fatty acid ester selected from the group consisting of Polysorbate 20 and Polysorbate 80.

9. The granulocyte colony-stimulating factor-containing formulation of Claim 1, which has a pH of 5-7.

10. The granulocyte colony-stimulating factor-containing formulation of Claim 1, which has a pH of 6-6.8.

11. The granulocyte colony-stimulating factor-containing formulation of Claim 1, which has a pH of 6.2-6.8.

12. The granulocyte colony-stimulating factor-containing formulation of Claim 1, which is packed in a vial, ampoule or prefilled syringe.

ABSTRACT

A stable granulocyte colony-stimulating factor-containing formulation comprising a granulocyte colony-stimulating factor and at least one pharmaceutically acceptable surfactant in an amount of 1 part by weight or less per part by weight of the granulocyte colony-stimulating factor and having a pH of 7 or less.

Fig. 1

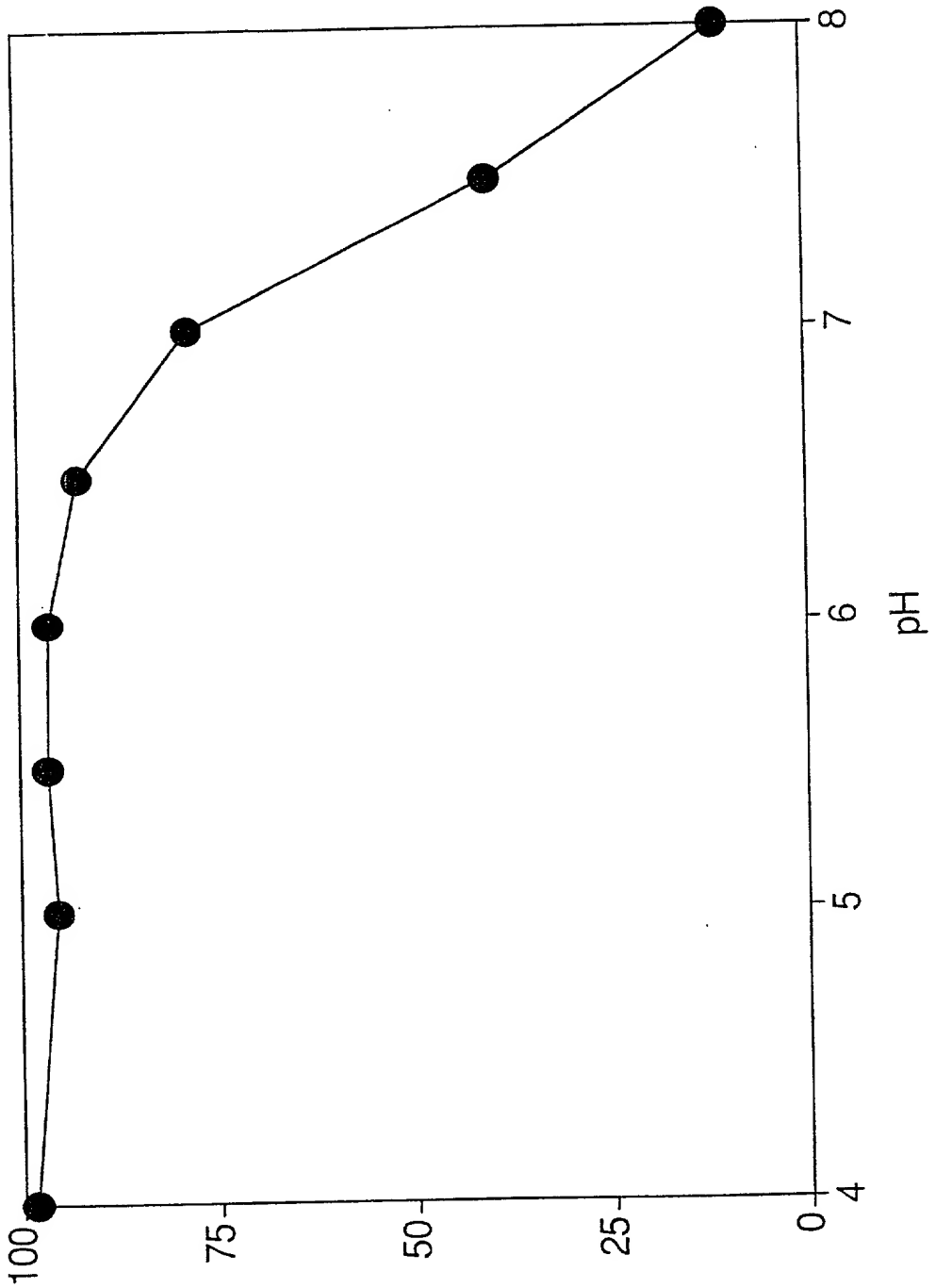
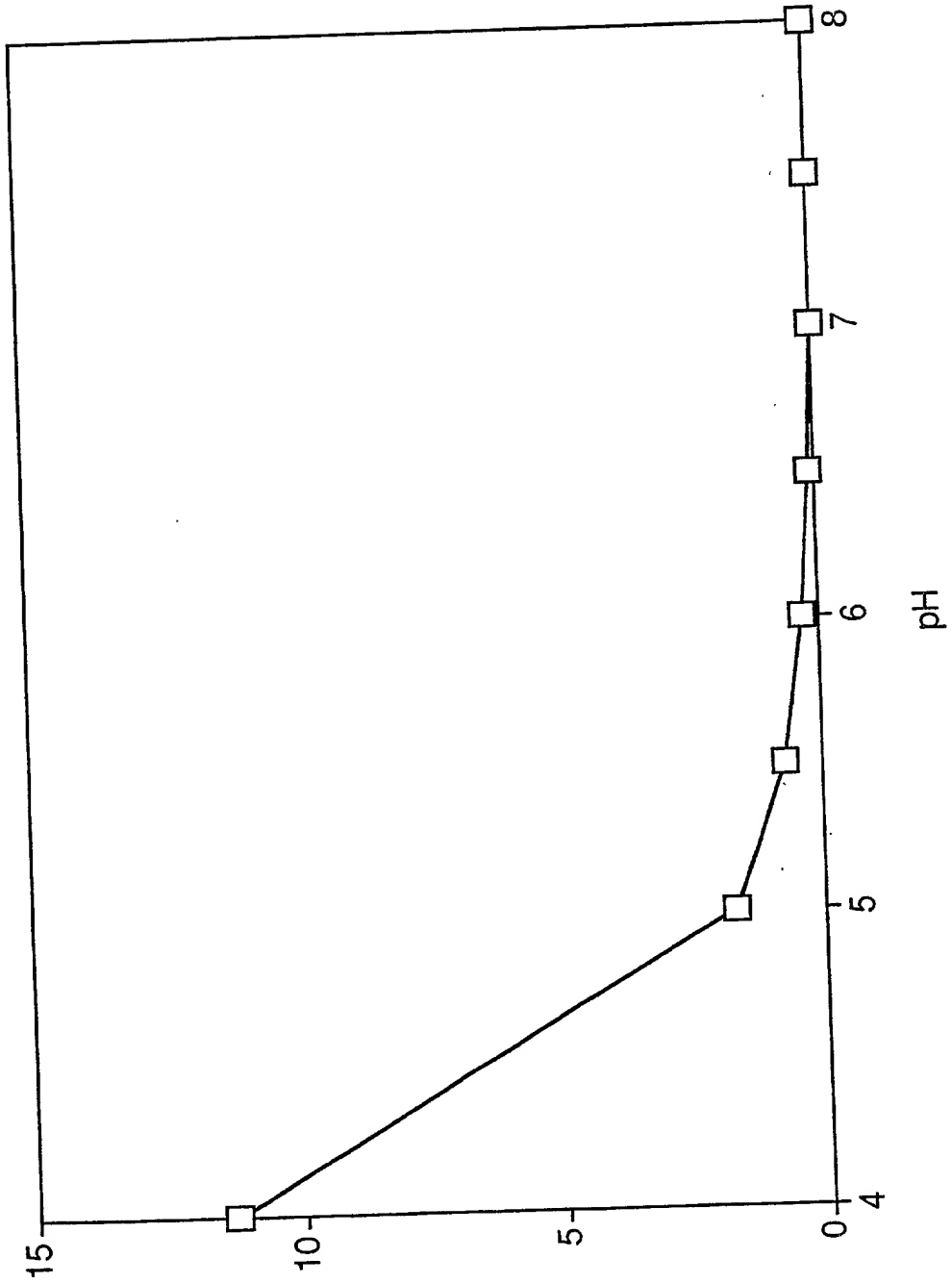
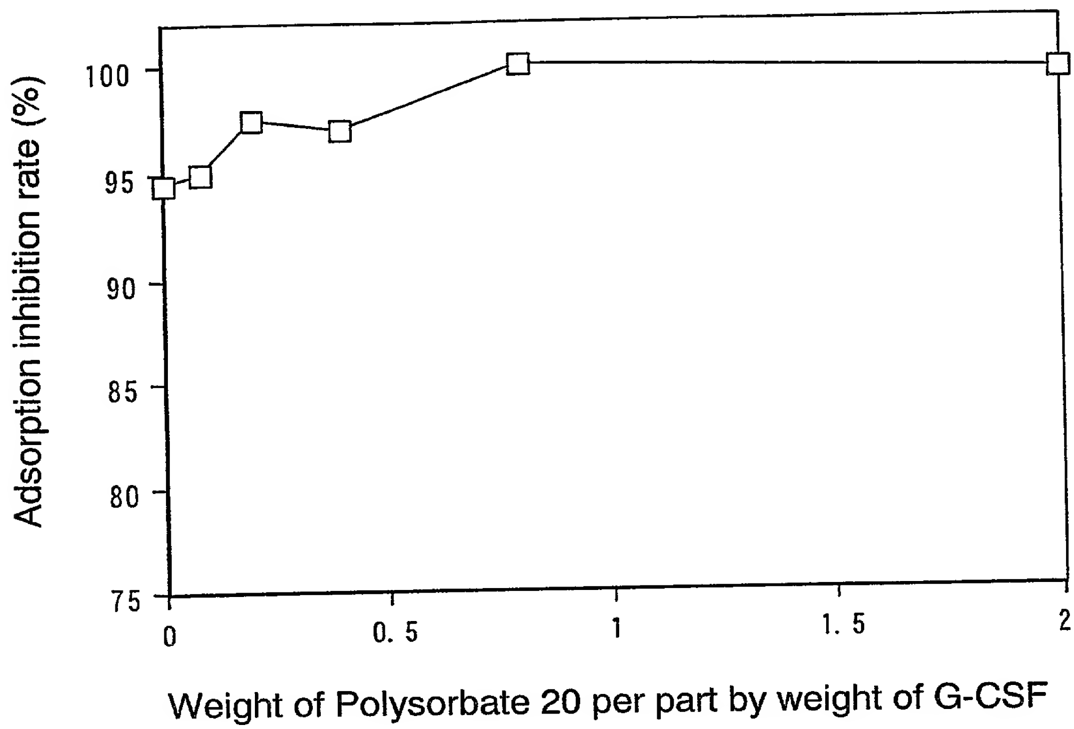

8
7
6
5
4
pH
100
75
50
25
0
Percentage (%) of remaining G-CSF
after acceleration at 40 °C for 2 weeks

Fig. 2



Content (%) of desialylated G-CSF
after acceleration at 40 °C for 2 weeks

Fig. 3



Docket No. _____

COMBINED DECLARATION AND POWER OF ATTORNEY FOR
ORIGINAL, DESIGN, NATIONAL STAGE OF PCT, SUPPLEMENTAL
DIVISIONAL, CONTINUATION OR CONTINUATION-IN-PART APPLICATION

As a below name inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PROTEIN-FREE FORMULATIONS

the specification of which

a. [X] is attached hereto

b. [ ] was filed on _____ as application Serial No. _____ and was amended on _____. (if applicable).

PCT FILED APPLICATION ENTERING NATIONAL STATE

c. [X] was described and claimed in International Application No. PCT/JP99/01080 filed on March 5, 1999 and as amended on _____. (if any).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

I hereby specify the following as the correspondence address to which all communications about this application are to be directed:

SEND CORRESPONDENCE TO: MORGAN & FINNEGAN, L.L.P
345 Park Avenue
New York, N.Y. 10154

DIRECT TELEPHONE CALLS TO: _____
(212) 758-4800

[X] I hereby claim foreign priority benefits under Title 35, United States Code § 119(a)-(d) or under § 365(b) of any foreign application(s) for patent or inventor's certificate or under § 365(a) of any PCT international application(s) designating at least one country other than the U.S. listed below and also have identified below such foreign application(s) for patent or inventor's certificate or such PCT international application(s) filed by me on the same subject matter having a filing date within twelve (12) months before that of the application on which priority is claimed:

[x] The attached 35 U.S.C. § 119 claim for priority for the application(s) listed below forms a part of this declaration.

Country/PCT	Application Number	Date of filing (day, month, yr)	Date of Issue (day, month, yr)	Priority Claimed
Japan	54799/1998	6/3/1998		[x] YES [ ] NO
				[ ] YES [ ] NO
				[ ] YES [ ] NO

[ ] I hereby claim the benefit under 35 U.S.C. § 119(e) of any U.S. provisional application(s) listed below.

Provisional Application No.	Date of Filing (day, month, yr)

ADDITIONAL STATEMENTS FOR DIVISIONAL, CONTINUATION OR CONTINUATION-IN-PART OR PCT INTERNATIONAL APPLICATION(S) (DESIGNATING THE U.S.)

I hereby claim the benefit under Title 35, United States Code § 120 of any United States application(s) or under § 365(c) of any PCT international application(s) designating the U.S. listed below.

US/PCT Application Serial No.	Filing Date	Status (patented, pending, abandoned)/ U.S. application no. assigned (For PCT)
US/PCT Application Serial No.	Filing Date	Status (patented, pending, abandoned)/ U.S. application no. assigned (For PCT)

[ ] In this continuation-in-part application, insofar as the subject matter of any of the claims of this application is not disclosed in the above listed prior United States or PCT international application(s) in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or Imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

I hereby appoint the following attorneys and/or agents with full power of substitution and revocation, to prosecute this application, to receive the patent, and to transact all business in the Patent and Trademark Office connected therewith: John A. Diaz (Reg. No. 19,550), John C. Vassil (Reg. No. 19,098), Alfred P. Ewert (Reg. No. 19,887), David H. Pfeffer, P.C. (Reg. No. 19,825), Harry C. Marcus (Reg. No. 22,390), Robert E. Paulson (Reg. No. 21,046), Stephen R. Smith (Reg. No. 22,615), Kurt E. Richter (Reg. No. 24,052), J. Robert Dailey (Reg. No. 27,434), Eugene Moroz (Reg. No. 25,237), John F. Sweeney (Reg. No. 27,471), Arnold I. Rady (Reg. No. 26,601), Christopher A. Hughes (Reg. No. 26,914), William S. Feiler (Reg. No. 26,728), Joseph A.

Calvaruso (Reg. No. 28,287), James W. Gould (Reg. No. 28,859), Richard C. Komson (Reg. No. 27,913), Israel Blum (Reg. No. 26,710), Bartholomew Verdirame (Reg. No. 28,483), Maria C.H. Lin (reg. No. 29,323), Joseph A. DeGirolamo (Reg. No. 28,595), Michael A. Nicodema (Reg. No. 33,199), Michael P. Dougherty (Reg. No. 32,730), Seth J. Atlas (Reg. No. 32,454), Andrew M. Riddles (Reg. No. 31,657), Bruce D. DeRenzi (Reg. No. 33,676), Michael M. Murray (Reg. No. 32,537) and Mark J. Abate (Reg. No. 32,527) of Morgan & Finnegan, L.L.P. whose address is: 345 Park Avenue, New York, New York, 10154; and Edward A. Pennington (Reg. No. 32,588) of Morgan & Finnegan, L.L.P., whose address is 1775 Eye Street, Suite 400, Washington, D.C. 20006.

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[x] I hereby authorize the U.S. attorneys and/or agents named hereinabove to accept and follow instructions from YUASA AND HARA as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. attorneys and/or agents and me. In the event of a change in the person(s) from whom instructions may be taken I will so notify the U.S. attorneys and/or agents hereinabove.

1-00

Full name of sole or first inventor Shuji SUMIDA

Inventor's signature* Shuji Sumida July 26, 2000

Shuji SUMIDA date

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Citizenship Japanese

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2-00

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Inventor's signature* Yasushi Sato July 26, 2000

Yasushi SATO date

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Citizenship Japanese

Post Office Address c/o Chugai Seiyaku Kabushiki Kaisha of 41-8, Takada 3-chome, Toshima-ku, Tokyo 171-8545 Japan

[ ] ATTACHED IS ADDED PAGE TO COMBINED DECLARATION AND POWER OF ATTORNEY FOR SIGNATURE BY THIRD AND SUBSEQUENT INVENTORS FORM.

* Before signing this declaration, each person signing must:

1. Review the declaration and verify the correctness of all information therein; and

2. Review the specification and the claims, including any amendments made to the claims.

After the declaration is signed, the specification and claims are not to be altered.

To the inventor(s):

The following are cited in or pertinent to the declaration attached to the accompanying application:

Title 37, Code of Federal Regulation, § 1.56

Duty to disclose information material to patentability.

(a) A patent by its very nature is affect with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is canceled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is canceled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in patent was cited by the Office or submitted to the Office in the manner prescribed by §§1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

(1) prior art cited in search reports of a foreign patent office in a counterpart application, and

(2) the closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.

Title 35, U.S. Code § 101

Inventions patentable

Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

Title 35 U.S. Code § 102

Conditions for patentability; novelty and loss of right to patent

A person shall be entitled to a patent unless –

(a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for patent,

(b) the invention was patented or described in a printed publication in this or foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States, or

(c) he has abandoned the invention, or

(d) the invention was first patented or caused to be patented, or was the subject of an inventor's certificate, by the applicant or his legal representatives or assigns in a foreign country prior to the date of the application for patent in this country on an application for patent or inventor's certificate field more than twelve months before the filing of the application in the United States, or

(e) the invention was described in a patent granted on an application for patent by another filed in the United States before the invention thereof by the applicant for patent, or on an international application by another who has fulfilled the requirements of paragraphs (1), (2), and (4) of section 371(c) of this title before the invention thereof by the applicant for patent, or

(f) he did not himself invent the subject matter sought to be patented, or

(g) before the applicant's invention thereof the invention was made in this country by another had not abandoned, suppressed, or concealed it. In determining priority of invention there shall be considered not only the respective dates of conception and reduction to practice of the invention, but also the reasonable diligence of one who was first to conceive and last to reduce to practice, from a time prior to conception by the other ...

Title 35, U.S. Code § 103

Conditions for patentability; non-obvious subject matter

A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

Subject matter developed by another person, which qualifies as prior art only under subsection (f) or (g) of section 102 of this title, shall not preclude patentability under this section where the subject matter and the claimed invention were, at the time the invention was made, owned by the same person or subject to an obligation of assignment to the same person.

Title 35, U.S. Code § 112 (in part)

Specification

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise and exact terms also enable any person skilled in the art to which it pertains, or with which it is mostly nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Title 35, U.S. Code § 119

Benefit of earlier filing date in foreign country; right of priority

An application for patent for an invention filed in this country by any person who has, or whose legal representatives or assigns have, previously regularly filed an application for a patent for the same invention in a foreign country which affords similar privileges in the case of applications filed in the United States or to citizens of the United States, shall have the same effect as the same application would have if filed in this country on the date on which the application for patent for the same invention was first filed in such foreign country, if the application in this country is filed within twelve months from the earliest date on which such foreign application was filed; but no patent shall be granted on any application for patent for an invention which had been patented or described in a printed publication in any country more than one year before the date of he actual filing of the application in this country, or which had been in public use or on sale in this country more than one year prior to such filing.

Title 35, U.S. Code § 120

Benefit or earlier filing date in the United States

An application for patent for an invention disclosed in the manner provided by the first paragraph of section 112 of this title in an application previously filed in the United States, or as provided by section 363 of this title, which is filed by an inventor or inventors named in the previously filed application shall have the same effect, as to such invention, as though filed on the date of the prior application, if filed before the patenting or abandonment of or termination of proceedings on the first application or an application similarly entitled to the benefit of the filing date of the first application and if it contains or is amended to contain a specific reference to the earlier filed application.

Please read carefully before signing the Declaration attached to the accompanying Application.

If you have any questions, please contact Morgan & Finnegan, L.L.P.

FORM:COMB-DEC.NY
Rev. 5/21/98